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A genetically confirmed hybrid between *Natrix astreptophora* and *N. maura*, two deeply divergent and ancient snake species

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Abstract

Using two mitochondrial DNA fragments and six nuclear genes, we confirmed that a morphologically intermediate snake, captured in Andalusia, southwestern Spain (municipality of Huelva), was an F1 hybrid between a female *Natrix astreptophora* and a male *N. maura*. The two parental species diverged 21.5 million years ago, which is why their capability to hybridize is remarkable. The interspecific mating could have been facilitated by the rarity of *N. astreptophora* and the abundance of *N. maura* in southwestern Spain, making it difficult for *N. astreptophora* females to find conspecific males. In the face of previously published genomic signatures for ancient gene flow between *N. astreptophora* and *N. maura*, our finding raises the question of whether occasional hybridization still contributes to a continued exchange of genetic information between these two deeply divergent and old taxa.

Key Words

Huelva, hybridization, molecular genetics, Natricidae, Spain

The genus *Natrix* comprises five species (Speybroeck et al. 2020). Three taxa are semiaquatic — *Natrix astreptophora* (Seoane, 1884), *N. helvetica* (Lacepède, 1789), and *N. natrix* (Linnaeus, 1758), which were long assumed to be conspecific (Kabisch 1999; Pokrant et al. 2016; Kindler et al. 2017), while the remaining two are highly aquatic — *N. maura* (Linnaeus, 1758) and *N. tessellata* (Laurenti, 1768). However, despite many ecological similarities and a superficial morphological resemblance, the two aquatic species are not sister taxa (Guicking et al. 2006). As confirmed by a dated phylogeny using 430 genome fragments of 320 kb length (Schöneberg et al. 2023), *N. maura* is sister to all other *Natrix* species, and *N. tessellata* is the sister species of a crown clade comprised of *N. natrix* + (*N. astreptophora* + *N. helvetica*). Hybrids between most *Natrix* species have been reported and confirmed genetically (reviewed in Schöneberg et al. 2023). Crosses involving *N. maura* are rare. Although there are genomic footprints of past gene flow between *N. maura* and all other *Natrix* species except *N. tessellata* (Schöneberg et al. 2023), only three putative hybrids with the sympatric *N. astreptophora* have been described to date, based on morphology (González de la Vega et al. 2021).

The distribution ranges of *N. astreptophora* and *N. maura* widely overlap, and the species co-occur in the North African Maghreb Region, the Iberian Peninsula, and southwestern France (Geniez 2015). The putative hybrids were captured at three different sites in Andalusia, southern Spain (provinces of Huelva, Málaga, and Sevilla), and had a combination of external traits characteristic of the supposed parental species (González de la Vega et al. 2021). Only one of the putative hybrids was sampled

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for genetic investigation. In the present study, we genetically examined this individual and compared it to its putative parental species (Fig. 1) to determine whether it indeed represents a hybrid.

Tissue samples (scales) of the morphologically intermediate snake and one representative each of *N. astreptophora* and *N. maura* were collected and stored in pure ethanol; the snakes were released after sampling at the collection sites. The putative hybrid (municipality of Huelva, 37°18'12"N, 6°52'29"W) and the *N. maura* (La Mediana, Almonte, 37°07'18"N, 6°36'38"W) originate from the province of Huelva, and the *N. astreptophora* (Cazalla de la Sierra, 37°56'39"N, 5°44'12"W) is from the neighboring province of Sevilla. Two frequently used mitochondrial DNA (mtDNA) fragments were sequenced, the cyt *b* gene and a segment comprising the partial ND4 gene plus adjacent DNA coding for tRNAs (Kindler et al. 2013, 2017; Pokrant et al. 2016). As nuclear markers, six loci with species-specific alleles were selected: the BDNF (Vieites et al. 2007), Cmos (Lawson et al. 2005), MC1R (Pinho et al. 2010), NT3, PRLR (both: Townsend et al. 2008), and Rag-1 genes (Groth and Barrowclough 1999). Laboratory procedures followed the general workflow described in Kindler et al. (2013); for PCR conditions, primer sequences, annealing temperatures, and fragment lengths, see Suppl. material 1: table S1. Sequences were edited and aligned using BIOEDIT 7.0.5.2 (Hall 1999). Nuclear sequences were phased using DnaSP 6 (Rozas et al. 2017) before analysis. For each gene, parsimony networks were drawn using TCS 1.21 (Clement et al. 2000) and POPART 1.7 (Leigh and Bryant 2015).

The genetic analyses confirmed that the morphologically intermediate snake is a hybrid of *N. astreptophora* and *N. maura*. The networks show that the mitochondrial haplotypes (Fig. 2 top) of the two species are highly distinct and differ in 140 (cyt *b*) and 53 mutations (ND4+tRNAs). The hybrid shares either the haplotype of the *N. astreptophora* (ND4+tRNAs) or has a haplotype differing in one mutation step from that of the *N. astreptophora* (cyt *b*). For each of the six nuclear loci (Fig. 2, center and bottom), alleles of the two species are present in the hybrid. For the BDNF, MC1R, and NT3 genes, the respective alleles of *N. astreptophora* and *N. maura* are separated by 3–10 mutations, and the hybrid has the same alleles as each of the supposed parental species. For Cmos, the hybrid shares one allele with the *N. astreptophora* individual; its second allele differs by one mutation step from each of the two alleles of the *N. maura*. The alleles of the pure *N. astreptophora* and *N. maura* are separated by a minimum of 3 or 4 steps. For PRLR, the hybrid shares one allele with the *N. maura* sample, and its other allele differs in three mutations from that of the *N. astreptophora*, while the alleles of the pure representatives of the two species differ by 16 mutations. For the Rag-1 gene, the hybrid shares one allele with the *N. maura*; its second allele differs by one mutation from the allele of the *N. astreptophora*. The alleles of the two pure snakes are separated by six steps.

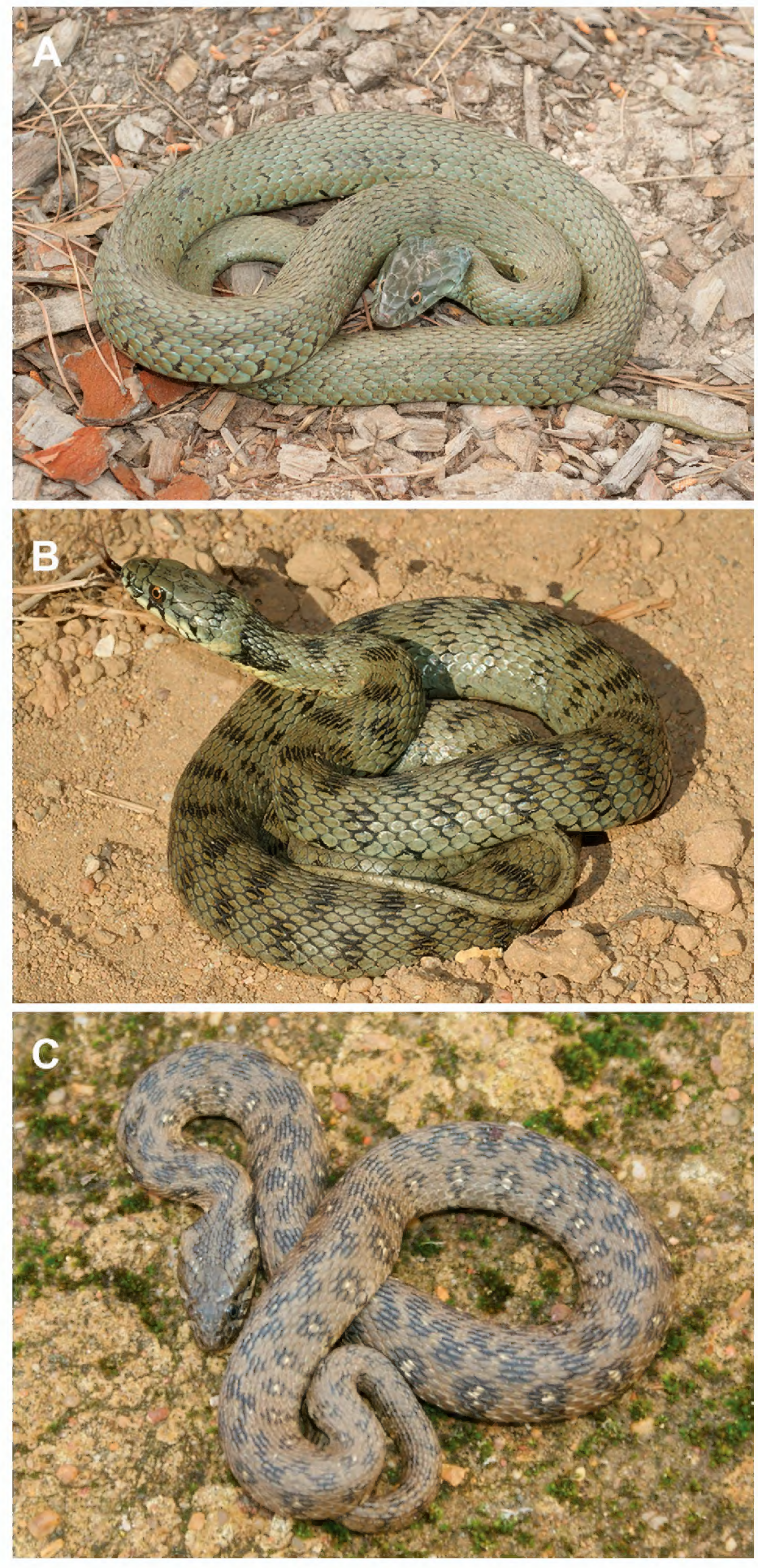

Figure 1. The putative hybrid *Natrix astreptophora* × *N. maura* and representatives of the parental taxa from Andalusia, Spain: **A.** *N. astreptophora*; **B.** Morphologically intermediate individual from the municipality of Huelva; **C.** *N. maura*. For a detailed morphological comparison, see González de la Vega et al. (2021). Photos: Juan Pablo González de la Vega.

There is no evidence of recombination in any of the six nuclear loci, suggestive of an F1 hybrid. Since the hybrid carries mitochondrial haplotypes of *N. astreptophora*, and mtDNA is inherited in the maternal line (Ballard and Whitlock 2004), its mother was a *N. astreptophora*. Together with *Coronella austriaca*, *N. astreptophora* is one of the two rarest snake species in Andalusia, while *N. maura* is very abundant there (González de la Vega 1988; González de la Vega et al. 2021). This rarity of *N. astreptophora* may have facilitated the mismatched mating because *N. astreptophora* females might have difficulties in finding conspecific males.

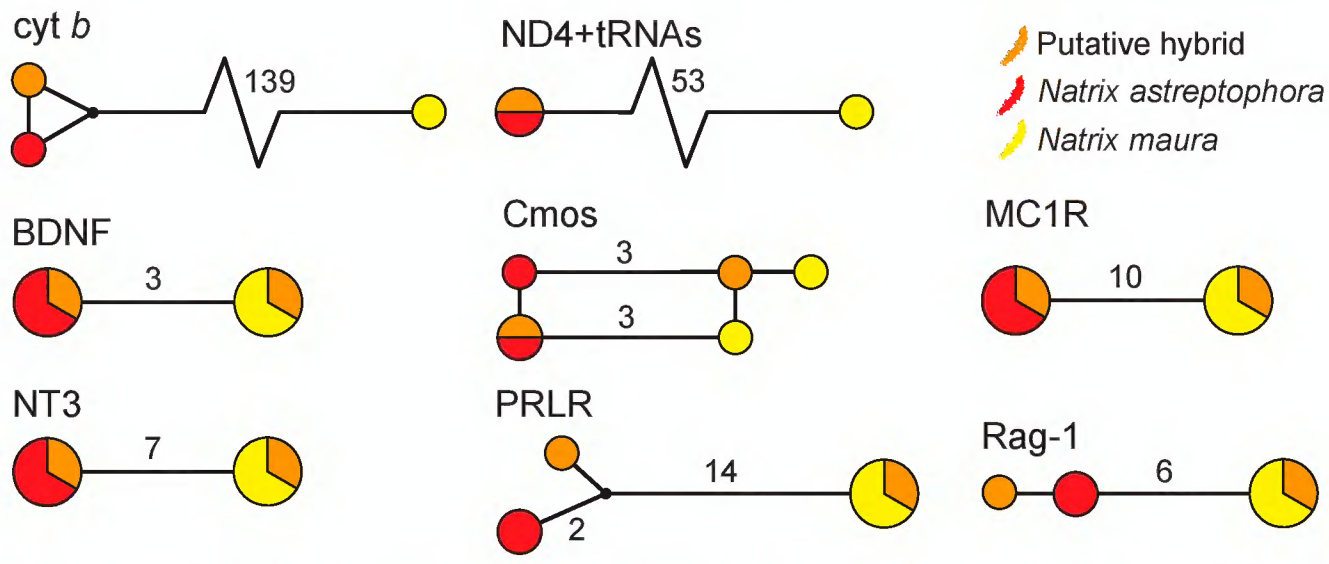

Figure 2. Parsimony networks for two mitochondrial (cyt *b*, ND4+tRNAs) and six nuclear (BDNF, Cmos, MC1R, NT3, PRLR, Rag-1) DNA fragments from the putative hybrid and its assumed parental species. Nuclear sequences were phased; symbol size corresponds to haplotype/allele frequency. Connecting lines represent one mutation step, if not otherwise indicated. Small black circles are missing node haplotypes/alleles.

Based on morphology, González de la Vega et al. (2021) speculated that also the two unsampled intermediate snakes were F1 hybrids. All three intermediate individuals were adults and looked healthy. It is unknown whether backcrosses or further hybrid generations are viable. According to a recently published fossil-calibrated molecular clock based on genomic data, *N. maura* diverged already in the early Miocene (21.5 million years ago) from the other *Natrix* species, even though there are genetic signatures for ancient hybridization with all other *Natrix* species, except *N. tessellata*, suggestive of gene flow between *N. maura* and the last common ancestor of *N. astreptophora*, *N. helvetica*, and *N. natrix* (Schöneberg et al. 2023). The genetically confirmed *N. astreptophora* × *N. maura* hybrid raises the question of whether there is still limited and continued exchange of genetic information between the two species, as it has been shown for *N. tessellata* and *N. natrix* (Asztalos et al. 2021), two taxa that diverged approximately three million years later (Schöneberg et al. 2023).

European Nucleotide Archive accession numbers for the nuclear DNA sequences generated in the present study are OZ206094–OZ206119 (BioProject PRJEB82314). The cyt *b* sequence of the *N. astreptophora* is a new haplotype differing in one step from the previously identified haplotype aE1 present in the hybrid. Following the haplotype nomenclature of Kindler et al. (2018) and Asztalos et al. (2020), the new haplotype receives the code aE26 (accession number OZ205784); all other mtDNA haplotypes have been previously identified.

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Supplementary material 1

Studied DNA sequences, primers, and thermocycling conditions

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Data type: xlsx

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